25. A nucleic acid probe comprising the sequence of one of SEQ ID NO. 1-4, capable of distinguishing between species of Shigella in a hybridization assay, or capable of distinguishing between Shigella and E. coli in a hybridization assay.--

Remarks

Applicant requests reconsideration and timely notice of allowance of all the pending claims.

The new claims are submitted to clarify the subject matter to methods for discriminating between and among *Shigella* and *E. coli*. No new matter enters by the amendments.

The Examiner indicated that unexpected results support the patentability of this subject matter (*see* pages 7-8 of Paper No. 12). As shown below, there is no need to limit the claims to a specific hybridization scheme. The evidence provided shows that the new claims are unobvious and that the Examiner's concerns do not effect the obviousness inquiry or the conclusion of unobviousness.

The Examiner concludes that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to construct the DNA sequences of the claimed invention for the use of probes and primers that could distinguish *Shigella* from *E. coli*. The inventor respectfully disagrees with the Examiner for the reasons discussed below.

First, a recent publication (Sabat, *et al.*) shows that unexpectedly higher temperatures are needed for the separate nucleic acid-based identification of these species - temperatures higher than T_m. Second, all the evidence proves that one of ordinary skill in the art has repeatedly tried and failed to use DNA sequences to separately identify these species. Third, the references cited by the PTO specifically provide art that *fails* to teach the invention of Portugal.

A. New Document Sabat, *et al.* Proves the New Claims are Unobvious

Sabat, et. al ("Selective and Sensitive Method for PCR Amplification of *Escherichia coli* 16S rRNA Genes in Soil," *Appl. Environ. Microbiol.* 66:844-849, 2000) discuss how to discriminate *E. coli* from *Shigella* with the use of the polymerase chain reaction (PCR). This could be done if the "selective amplification of *E. coli* occurred only when the annealing

temperature in the PCR was elevated to 72° C, *which is 10° C higher than the optimum for the primers*" (see abstract on page 844).

The authors conclude, "To the best of our knowledge, this is the first report of a PCR protocol based on amplification of 16S rRNA that effectively distinguishes *E. coli* from these closely related bacteria" (page 849, paragraph 2).

The clear and inescapable conclusion from Sabat, *et al.* is that hybridization protocols could not, at the time this application was filed, be used to discriminate between and among *Shigella* and *E. coli* species. The amended claims, therefore, cannot be obvious over any combination of earlier hybridization art and certainly not over the cited art.

The findings of Sabat, *et al.* also prove that the invention of Portugal is independent of both sequence and process for discriminating between and among *E. coli* and *Shigella* species.

The rRNA derived primers used by Sabat, *et al.* differ from those of Portugal (SEQ ID NO:4). The sequence of Portugal contains an extra T in the seventh position from the 5' end. This effectively alters the reading frame from positions 8-21. Comparing the Sabat, *et al.* and Portugal sequences, ten of the bases from positions 8-21 are mismatched. Ten mismatched bases of a 21-mer probe would be expected by one of ordinary skill in the art to react completely different in either system. The inventive concept of Portugal, using temperatures at or above the T_m, is what makes both of these assays work.

B. Prior Methods to Distinguish Between and Among *E. coli* and *Shigella* Have Clearly Failed

Second, investigators since at least 1988 have sought nucleic acid-based detection schemes to identify *E. coli* and *Shigella*. For example, Kyriaki Parodos *et al.* filed an application titled "Probes for the Specific Detection of *Escherichia coli* and Shigella," in 1988. The patent issued in 1992 as No. 5,084,565. This, however, fails to teach how to separately identify each species. Similarly, Hogan (cited by the Examiner and discussed below) ten years later (patent 5,714,321 issued February 3, 1998) still cannot teach how to discriminate between these very closely related species (the discussion of Hogan, below, proves this).

Investigators have particular incentive to demonstrate that their art can differentiate between species of *Shigella* and *E. coli* and/or among the separate species of Shigella. Reporting of Shigella cases is required by the federal government's Centers for Disease Control (CDC). Not only must each case be reported but also the particular infectious species of Shigella must be determined. Cases of Shigella in the United States and elsewhere is on the rise and, in the case of *S. dysentery* and *E. coli* O157:H7, can be life-threatening. *Shigella* is also a serious pathogen that has affected food safety and for which the Food and Drug Administration has issued a detailed protocol for testing. Tens of thousands of cases are being reported annually to the CDC, yet the commonly used techniques for detection and identification remain arduous and laborious.

So vexing has the problem of differentiating *Shigella* species and differentiating *Shigella* from *E. coli* become that investigators have concluded that "differentiation of *Shigella* from *E. coli* can prove one of the most difficult problems for a laboratory and may reflect the fact that *E. coli* and all four *Shigella* species are really the 'same species' on the basis of their close relatedness by DNA-DNA hybridization" (J.J. Farmer and M.T. Kelly, "Enterobacteriacea," in A. Balows et al. (Eds.), *Manual of Clinical Microbiology,* American Society for Microbiology, Washington, D.C., 1991, page 370).

C. The References Cited Fail to Show That the Claimed Invention is Obvious

1. Anderson

Anderson does not anticipate the invention of Portugal. Anderson teaches (page 17) that

> "as the length of the probe decreases, the T_m of the hybrid also decreases so that with some oligonucleotide probes the T_m may be so low that it is not feasible to carry out hybridization at *T*m-25° C. In practice hybridization with oligonucleotide probes is usually carried out *at 3-10° C below* T_m. *However, these conditions are very stringent...*"

Thus, Anderson clearly states that hybridization 3-10° C *below* the T_m is more than sufficient, whereas the invention of Portugal teaches that duplexes formed from oligonucleotides should be washed at temperatures that are either at the T_m or above it. Anderson does not teach or suggest <u>any</u> wash temperatures and certainly fails to teach or suggest the correct wash temperatures to use with individual oligonucleotides as in the claimed invention. In fact, she specifically states

(page 20) to wash at 5° C *below* the T_m when hybridizing with a mixture of oligonucleotides. Anderson, therefore, fails to teach or suggest washing at or above the T_m.

2. Hammond 5,374,718

Hammond also does not teach or suggest the invention of Portugal. Hammond explicitly teaches Column 4 (55-60) to choose probes that result "in a T_m about 2-10° C *higher* than the temperature at which the final assay will be performed." This means the conditions of Hammond are to hybridize at temperatures 2-10° C *below* the T_m. In Column 5 (10-15), Hammond explicitly teaches that *optimal* hybridization occurs "approximately 5° C *below* the melting temperature for a given duplex." Nowhere does Hammond teach or suggest the washing of a given duplex *at or above the* T_m of the probe.

In light of these explicit statements on temperature, when Hammond states (Column 4, 65-68) that "conditions such as ionic strength and incubation temperature under which a probe should be used should also be taken into account in constructing a probe," it is clear he is referring only to temperatures *below* the T_m. Nothing within the Hammond document mentions using any conditions *at or above* the T_m. Thus, Hammond fails to teach or suggest the claimed invention.

The Examiner asserts that Hammond discriminates between closely related organisms. However, even discriminating between species of Chlamydia does not imply that the present invention is obvious. While Hammond in his table (Columns 9 and 10) differentiates between *Clamydia pneumoniae* and closely related organisms, Hammond provides no data on the extent of mismatches in the rRNAs among those organisms. A comparison of the 16S rRNA sequences for *Chlamydia pneumoniae* (GenBank CHT16SR) and *Chlamydia psittaci* (GenBank E17341), two of the most closely related species in the table, reveals a far greater extent of mismatches in the region of interest in the 16S rRNA sequences than is seen between the four *Shigella* species or between *Shigella* and *E. coli*. Therefore, while Hammond may be sufficient to differentiate among the larger differences seen among species of *Chlamydia* and related organisms, it proves insufficient as Portugal teaches to differentiate the much more closely related species of *Shigella* and *E. coli*.

One should not extrapolate from these data of Hammond to the very subtle differences in rRNA sequences that distinguish *Shigella* species from *E. coli.*

3. Hogan 5,714,321

Hogan also *fails* to provide evidence of how to differentiate clearly between *Shigella* species or between each *Shigella* species and *E. coli*, with the exception of *Shigella dysenteriae.* Citing the Examiner's own reference to column 52 and Table 54, Hogan demonstrates that his *E. coli* probe *fails* to differentiate between *E. coli, Shigella flexneri, Shigella boydii,* and *Shigella sonnei.* In contrast, Portugal specifically teaches how to differentiate between *E. coli* and the four major species of *Shigella* as well as between *Shigella flexneri, Shigella boydii, Shigella sonnei,* and *Shigella dysenteriae*

In Column 7 (1-5), Hogan teaches the same art as Hammond, i.e., to hybridize the probe at temperatures 2-10° C *below the* T_m (see Hammond above). Hogan also teaches (Column 10, 15-20) that the "optimal hybridization for synthetic oligonucleotide probes of about 15-50 bases in length occurs approximately *5° C below* the melting temperature for a given duplex." Hogan therefore teaches that hybridizations should be conducted at the ideal temperature of 5° C *below* the T_m. Hogan does not teach anywhere in this patent to wash at temperatures *at or above* the T_m.

Furthermore, Hogan also fails to teach or suggest how to distinguish among closely related species including those of Legionella (Table 30), Streptococcus (Table 43), Pseudomonas (Table 44), Salmonella (Table 50) or *Shigella* and *E. coli* mentioned above. In contrast, Portugal teaches how to differentiate between five extremely closely related species.

4. Cilia

It would not be *prima facie* obvious to one of ordinary skill in the art to construct DNA sequences of the claimed invention that could distinguish *Shigella* from *E. coli.* While one could construct DNA probes, one could not anticipate or predict whether or not they would work. Distinguishing *Shigella* from *E. coli* is, in fact, precisely what Cilia is attempting to do in making his phylogenetic comparisons.

Cilia, however, fails to teach or suggest how to use rRNA sequences to differentiate between and among *E. coli* and *Shigella.* Despite analyzing sequence variations among *E. coli* and *Shigella* species, Cilia et al. state (page 456, right column, last paragraph) that "further analyses could not derive decisive phylogenetic ingroup relationships within the {*Escherichia* + *Shigella*}...clades, at least on the basis of SSU [small subunit] rRNA sequences analyses." Cilia also states (page 454, right column, third paragraph) that "*E. coli* and *Shigella* in fact belong to the same bacterial species because they share a genome relatedness greater than 70%. Cilia fails to teach or suggest how to differentiate between these species whereas Portugal successfully does through the use of temperature at or above the T_m, a result later confirmed by Sabat. Furthermore, Sabat and Portugal succeed despite the use of different probes and different analytical procedures. Sabat uses a technique taught by Portugal to distinguish between *Shigella* and *E. coli* by employing a PCR methodology. In contrast, Cilia, using PCR methodology but not the technique taught by Portugal, fails. These facts alone establish that the claimed invention of Portugal cannot be obvious over Cilia, any of the cited papers, or any combination of the cited papers.

For these reasons, new claims 19-25 are patentable and applicant requests a timely notice of allowance.

No other fees, extension of time fees, or requests for extension of time are believed to be necessary to enter and consider this paper. If, however, any extensions of time are required or any fees are due in order to enter or consider this paper or enter or consider any paper accompanying this paper, including fees for net addition of claims, applicant hereby requests any

extensions or petitions necessary and the Commissioner is hereby authorized to charge the credit card previously used for any fees.

Respectfully submitted,

Date: Jan. 22, 2001

By: _____
Applicant : Frank Portugal

Encls: Sabat et al.
Farmer and Kelly, page 370
U.S. Patent 5,084,565

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I certify that this document and its attachments (34 pages) is being transmitted to the Commissioner of Patents at fax number [(703) 305-3014] this 22 day of January, 2001.

Frank Portugal, Ph.D.
Application # 09/027,089